C2 Domain Function in Healthy and Diseased Brain

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Chapter 5
Purification of recombinant C2AB domains of Doc2B

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Abstract

Doc2B, a calcium sensor for spontaneous synaptic vesicle release, utilizes its two C2 domains to bind anionic phospholipids in response to a calcium signal in neurons. High purity protein preparation is essential in order to better characterize calcium- and lipid binding of the C2 domains, as well as to resolve their structure. Here we describe procedures for production and purification of double C2AB domains of Doc2B. GST fusion proteins were expressed in *E. coli*, cleaved with thrombin, and further purified by ion exchange and gel filtration chromatography, resulting in high purity preparation of Doc2B C2AB domains.

Introduction

Doc2B is a high-affinity calcium sensor for spontaneous synaptic vesicle release[1]. It consists of a MID domain and two C2 domains (C2A and C2B)[2]. The two C2 domains are responsible for calcium sensing and membrane binding, both critical for Doc2B function[1]. We have developed a purification method for C2AB domains of Doc2B. For this, we used as a starting point the purification of Synaptotagmin-1 (Syt-1), which has been extensively studied functionally and structurally for its role in the control of synaptic vesicle release[3-7]. C2AB domains of Syt-1 and Doc2B share 41% amino acid identity, and have closely related but distinct functions. Synaptotagmin-1 and Doc2B regulate different modes of neurotransmitter release in the mammalian brain by functioning as calcium sensors to trigger synaptic vesicle fusion. They bind calcium and anionic phospholipids such as phosphatidylserine (PS) via conserved amino acids in their calcium-binding pocket[8] and bind phosphatidylinositol (4,5)-bisphosphate (PIP_2) and the SNARE complex proteins in a calcium-independent manner, via their polybasic stretch[9]. All these interactions appear to contribute to proper timing and calcium-dependence of the synaptic vesicle fusion. Synaptotagmin-1 is essential for fast synchronous neurotransmitter release induced by action potentials and calcium influx into the presynaptic terminal[10]. It is anchored to the surface of synaptic vesicles via its transmembrane domain, and binds to anionic lipids half-maximally at calcium concentrations of 10-40 μM in the presence of PS-containing membranes[11]. Doc2B is a soluble protein that moves between the cytoplasm and the plasma membrane, and is activated to bind anionic lipids half-maximally at 175 nM calcium in living cells[1,12,13]. Mice lacking Doc2B exhibit a specific defect in spontaneous, but not evoked neurotransmission[1]. Other proteins containing two or more tandem C2 domains are required for calcium-dependent synaptic vesicle fusion in other systems (such as otoferlin in the inner hair cells in the auditory system[14]), and are believed to function in a fashion analogous to Synaptotagmin-1 and Doc2B[15].

Here we describe a three-step purification strategy for the C2AB domains of Synaptotagmin-1 and Doc2B. After initial affinity purification via the GST tag and its cleavage by thrombin, we used ion exchange chromatography to separate proteins by charge. As last step in the purification protocol we used gel filtration (size exclusion) chromatography to separate proteins by size and obtain a homogeneous protein preparation.

Results

Synaptotagmin-1

As the starting point for developing the purification method for DOC2B C2AB domains and control for our purification procedures, we first purified Synaptotagmin-1 C2AB domans. We used the published protocol for Syt-1 C2AB purification [16]. Details of purification are presented in the Materials and Methods section. In short, the Syt-1 C2AB fragment fused with an N-terminal GST tag was produced in *E. coli*, affinity purified on glutathione beads, and the GST tag was cleaved off with thrombin to produce soluble C2AB protein. At this stage, the yield of Synaptotagmin-1 C2AB domains was ~600 µg per liter of bacterial culture. After thrombin cleavage, C2AB domains were dialyzed into the buffer for the ion exchange purification. This step was performed on a weak cation exchanger HiTrap, with linear NaCl gradient from 25 to 500 mM. Synaptotagmin-1 eluted at approximately 350 mM NaCl (Fig. 1A). SDS-PAGE image showed presence of two protein fragments (Fig. 1A, inset). Expected molecular weight was 36 kDa, corresponding to the top fragment on the gel. An additional shortened fragment of Syt-1 co-purified with the original C2AB fragment, possibly due to cleavage of either C-terminal or N-terminal unstructured regions. Subsequent gel permeation purification step using Superdex75 column did not completely separate the two protein fragments (Fig. 1B). However, this purification step substantially increased protein purity (table 1). The resulting protein fragments remained stable when stored at 4°C for up to two days, and after one freeze-thaw cycle. Based on these results, we proceeded with the purification of Doc2B C2AB domains.

Table 1. Synaptotagmin-1 and Doc2B C2AB domains purity at each purification step

Purification step	Synaptotagmin-1	Doc2B
Affinity	38%	49%
Ion exchange	52%	55%
Size exclusion	72%	>80%

Doc2b

Compared to Synaptotagmin-1, the C2AB fragment of Doc2B produced a lower yield per volume of bacterial culture (~300 µg/l for Doc2B). Thus in the first instance we focused on improving the yield of DO2B C2AB domains. To test if the yield and stability of the protein was affected by different N-terminal truncations of the C2AB fragment we compared the expression of two Doc2B constructs (amino acid residues 115-412 and 121-412, NP_112404). However, we did not observe any detectable difference in the yield or stability of these two constructs (data not shown).

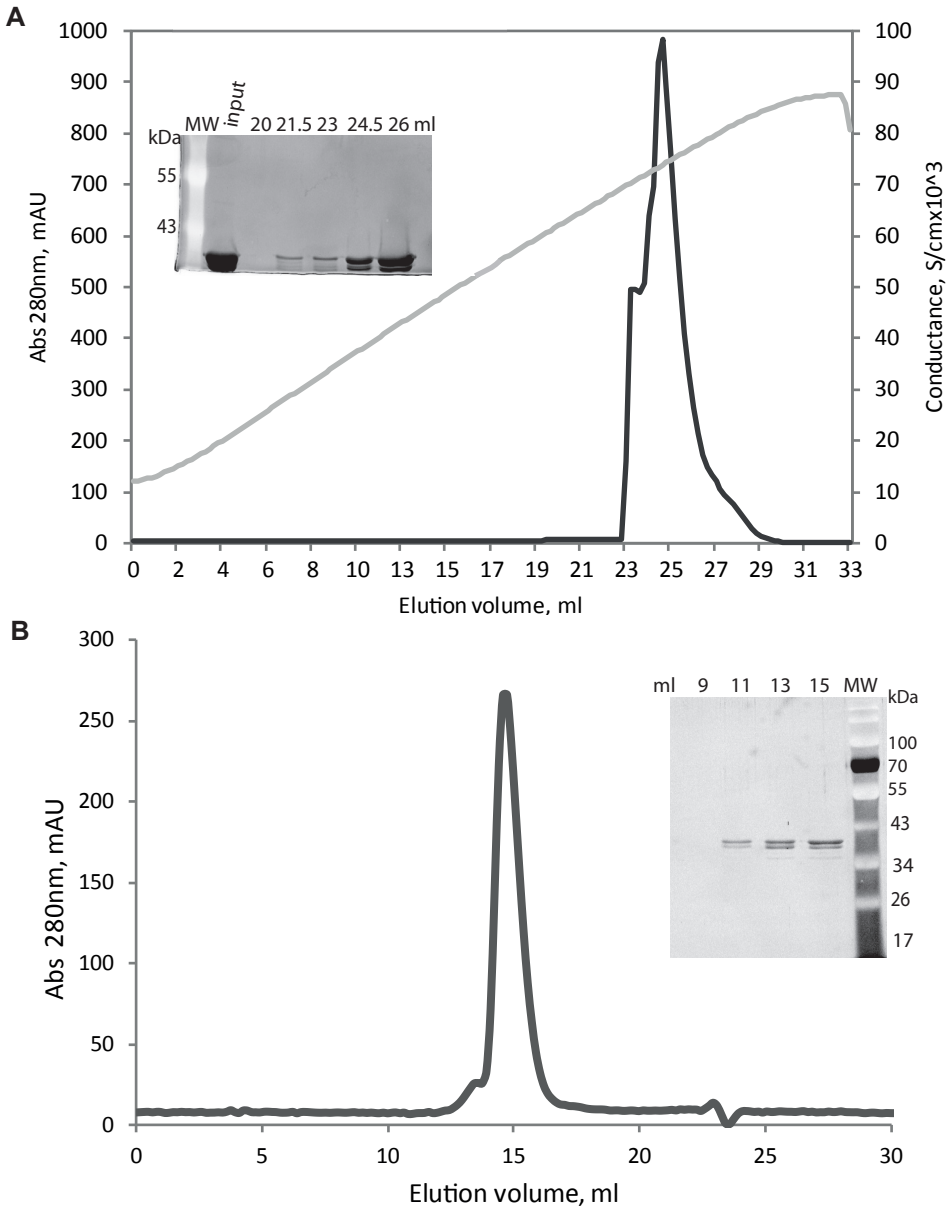

Figure 1. Purification of Synaptotagmin-1 C2AB (aa. 96-421) by ion exchange and size exclusion chromatography. A. Ion exchange (HiTrap Heparin HP column) purification of Synaptotagmin-1: chromatogram showing UV absorption of eluted fractions, inset: SDS-gel electrophoresis of peak fractions (1% of fraction volume loaded on gel, stained with Sypro Ruby) **B.** Size exclusion (Superdex75 column) purification of Synaptotagmin-1: chromatogram and gel of peak fractions (1% of fraction volume loaded on gel).

To optimize the conditions of growth, we tested whether expression of either Doc2B constructs improved if the induction of the *lac* promoter was done at 18°C overnight with 100 µM IPTG, rather than at 37°C with 500 µM IPTG for 4 hours. These conditions slow down the rate of protein synthesis, allowing more accurate folding. Again there was no significant difference in Doc2B yield (data not shown).

We used the Rosetta *E. coli* strain, designed to enhance the expression of eukaryotic proteins that contain codons rarely used in bacteria (this strain is modified to produce tRNAs in proportional quantities typical for a eukaryotic cell). Transforming expression constructs into Rosetta *E. coli* increased the yield of Doc2B C2AB domains by about 50% (from 300 to approximately 450 µg/l of bacterial culture).

Normally the *E. coli* cultures are grown in presence of 2% glucose to the growth medium to block leaky expression (before induction with IPTG). However in our hands, omission of glucose did not affect the yield of Doc2B, and for our protein production we did not add glucose. When growing bacterial cultures and collecting bacterial pellets on the same day, we used freshly prepared (not autoclaved) growth medium.

After immobilization on a glutathione affinity column, followed by thrombin cleavage, we used cation exchange chromatography as a next step. Doc2B did not bind strongly to the HiTrap resin used for Syt-1 (not shown), while ResourceS proved highly suitable for purification of Doc2B C2AB domains (Fig. 2A). In the linear NaCl gradient from 25 to 500 mM, Doc2B C2AB domains eluted at approximately 180 mM NaCl. As a final purification step, we used Superdex75 gel filtration column to optimize homogeneity of the protein sample. Both the high and low molecular weight impurities were removed during this step (Fig. 2B, inset). The sample eluted as a single peak without visible shoulders, indicating a homogenous protein preparation. Analysis of the band intensities obtained in gel electrophoresis indicated over 80% purity of the Doc2B C2AB preparation after the size exclusion purification step (Table 1). The purified C2AB domains of Doc2B were stable for at least 2 days when stored at 4°C (showing no degradation or reduction in quantity as determined by SDS-PAGE and Sypro Ruby staining). Doc2B activity (calcium-dependent lipid binding) was confirmed by liposome binding assay[17] following purification (Fig. 3).

Discussion

We observed substantial differences in the production and purification of recombinant C2AB domains of Doc2B and Synaptotagmin-1 from bacterial cultures, including yield and purification conditions, even if they share a high degree of sequence identity and have very similar functions. We observed a lower yield of Doc2B C2AB domains, compared to Synaptotagmin-1 C2AB domains. Of the different strategies used to increase the yield of Doc2B (different growth medium, time and temperature of expression induction, varying peptide chain length), only using the Rosetta strain of *E. coli* instead of the standard strain gave a significant improvement.

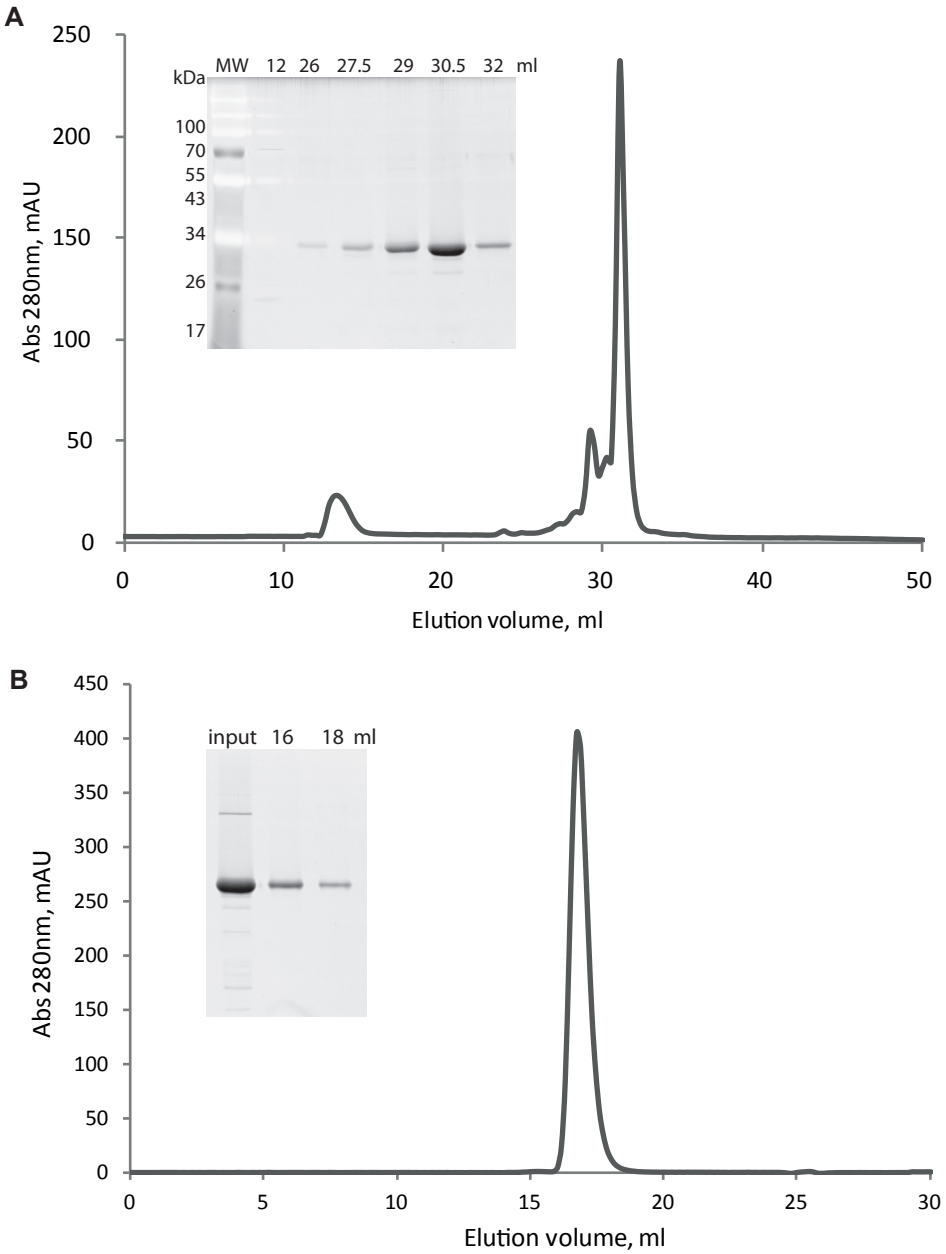

Figure 2. Purification of Doc2B C2AB (aa. 115-412) by ion exchange and size exclusion chromatography. A. Ion exchange (ResourceS column) purification of Doc2B: chromatogram showing UV absorption of eluted fractions, inset: SDS-gel electrophoresis of peak fractions (1% of fraction volume loaded on gel, stained with Sypro Ruby) **B.** Size exclusion (Superdex75 column) purification of Doc2B: chromatogram and gel of input and peak fractions (1% of fraction volume loaded on gel).

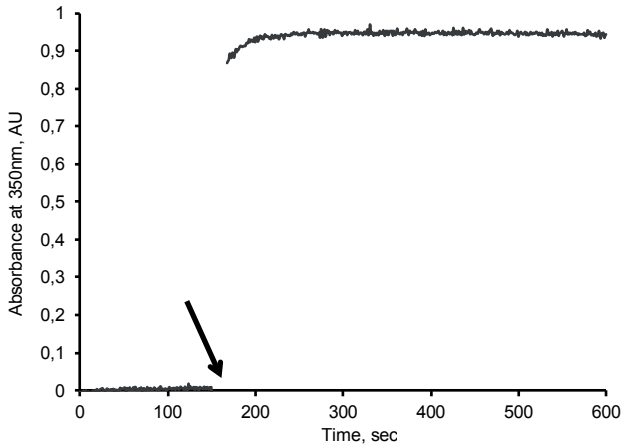

Figure 3. Activity of purified Doc2B C2AB domains. Purified Doc2B C2AB domains aggregated liposomes (lipid composition 25% phosphatidylserine, 75% phosphatidylcholine) in the presence of 1 mM calcium. First 150 seconds show the liposome baseline absorption, then addition of protein (arrow), and subsequent increase in absorption, proportional to liposome aggregation via C2AB domain cross-linking.

Because Rosetta strain is codon-enriched (producing tRNAs in a proportion that is typical for a eukaryotic cells), this suggests that the rat Doc2B sequence contains a higher percentage of codons that are rare in standard *E. coli* strains. As an alternative to the use of the Rosetta *E. coli* strain, another efficient solution would be to use codon-optimized DNA constructs for expression in standard bacterial strains. These are based on the same principle as the Rosetta strain, but in this case it is the DNA construct that is modified to fit the typical tRNA content of a bacterial cell. The amino acid sequence remains unchanged in both cases.

Although Synaptotagmin-1 C2AB domains purified as two fragments of similar molecular weight (and thus difficult to separate), Doc2B C2AB domains showed high purity on the SDS-PAGE, and a symmetric peak on the chromatogram.

E. coli is a suitable expression system for C2 domain production, even if it does not provide most post-translational modifications typical for mammalian proteins, such as phosphorylaton and glycosylation. To date, there is no evidence that post-translational modifications in C2 domains contribute to their function, although several phosphorylation sites have been reported in the C2A and C2B domains of Synaptotagmin-1[18].

We have developed a new protocol for purification of Doc2B C2AB domains, which can be applied to reproducibly obtain highly pure and stable recombinant protein. This can be used to further characterize membrane/lipid binding of DOC2B in *in vitro* assays, as well as structural and biophysical studies. Crystal structure of Synaptotagmin-1 C2AB domains has been resolved[19,20]. Comparing the structure and pinpointing the functional differences between Doc2B and Synaptotagmin-1 would improve our understanding of the mechanisms of neurotransmitter release.

Materials and methods

Rat Doc2B constructs (amino acid residues 115-412 and 121-412; NP_112404) were cloned into pGEX vector (GE Healthcare). Rat Synaptotagmin-1 construct (amino acid residues 97-421) in pGEX vector was kindly provided by Dr. Sascha Martens[21]. In all the constructs we introduced a Gly-Ser-Gly-Ser linker between GST and the C2 domain insert, that increased thrombin cleavage efficiency. These clones were heat-shock transformed (60 sec at 42°C) into *E. coli* BL21 DE3, or Rosetta DE3 pLysS (Merck) strain for expression. Cultures in medium supplemented with ampicillin were grown to OD600 0.6-0.8, and gene expression was induced with 0.5 mM IPTG at 37°C for 4 hours (or alternatively, with 0.1mM IPTG at 18°C for 15 hours).

Bacterial growth media used: LB (Luria-Bertani, BD), 2xTY (16 g Bacto-tryptone, 10 g yeast extract, 5 g NaCl in 1L MQ), supplemented with ampicillin (100 µg/ml).

Bacterial pellets were collected by centrifugation (30 mins, 4400 rpm at 4°C) and re-suspended in resuspension buffer (500 mM NaCl, 50 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4 NaOH, 5 mM β-mercaptoethanol, protease inhibitors (Sigma complete, without EDTA)), flash-frozen in liquid nitrogen, and stored at -80°C. To disrupt bacterial cells we sonicated the thawed lysates on ice (6 times for 30 seconds at 24 micron peak to peak), with at least 1 min break between each sonication cycle, in polypropylene 50 ml Falcon tubes filled to ~40 ml. Subsequently we solubilized the proteins by incubating the lysates at 4°C for 30-45 min in the presence of 1% Triton-X100. Finally, we centrifuged the lysates at 10000g for 30 minutes at 4°C, and incubated the supernatants with glutathione agarose beads (Sigma) to isolate the GST-fused protein. After 2-hour rotation with bacterial lysate at 4°C, the beads were washed with ice-cold high salt buffer (500 mM NaCl, 25 mM HEPES pH 7.4, 2 mM DTT), and treated with 10 µg/ml DNase I and RNAse A (both Roche, from bovine pancreas), in high salt buffer supplemented with 2 mM $MgCl_2$ for 20 minutes at room temperature.

Agarose beads with GST-C2AB fragments were incubated overnight at 4°C with thrombin (Serva), to cleave the linker between the GST and the C2 domains. Approximately 1 NIH unit for each 1µg GST fusion protein; thrombin cleavage buffer: 50 mM Tris pH 8.0, 150 mM NaCl, 3 mM $CaCl_2$, 2 mM DTT. After a short centrifugation at 1000rpm, solubilized Doc2B C2AB fragments were separated from the beads using a 300µl insulin syringe (Terumo) and exchanged into MES buffer (25 mM MES pH 6.0, 25 mM NaCl, 1 mM TCEP), while Synaptotagmin-1 C2AB fragments were exchanged into Hepes buffer (25 mM Hepes pH 7.4, 25 mM NaCl, 1 mM TCEP). Buffer exchange was performed using Pierce Dialysis cassettes (7 kDa molecular weight cut-off), at 4°C with constant mixing, for at least 4 hours. Before being loaded on the chromatographic column, all protein solutions were filtered using a 0.2 µm SFCA syringe filter (Corning).

The second purification step was performed on ion exchange column. DOC2B was purified by charge using a ResourceS cation-exchange column (GE Healthcare) on a

GE ÄKTA Purifier run at 1 ml/min in 25 mM MES (2-(N-morpholino)ethanesulfonic acid) NaOH pH 6.0 and 1 mM TCEP (Tris(2-carboxyethyl)phosphine), with a linear salt gradient from 25 to 500 mM NaCl (30 mins). For Synaptotagmin-1 ion exchange step we used HiTrap Heparin HP column (GE Healthcare), on a GE ÄKTA Purifier run at 1 ml/min in 25 mM Hepes NaOH pH 7.4 and 1 mM TCEP with a linear salt gradient from 25 to 500 mM NaCl. The flow-through fractions were analyzed by SDS-PAGE (15 µl of each 1.5 ml fraction loaded on gel) using Sypro Ruby (Biorad) staining and fluorescence imaging on FLA-5000 scanner (Fuji). Fractions with detectable protein levels were pooled together and concentrated using Centricon 10 kDa molecular-weight cut-off columns (Millipore), by centrifugation at 4400 rpm for approximately 20 mins (depending on initial volume) at 4°C in Sorvall RC-4 ultracentrifuge, swinging bucket rotor.

For the final purification step of both Synaptotagmin-1 and Doc2B C2AB domains we used gel filtration column (Superdex75, GE Healthcare) run at 0.6-0.7 ml/min on GE ÄKTA Purifier, for DOC2B in 25 mM MES pH 6.0, 500 mM NaCl and 1 mM TCEP, for Syt-1 in 25 mM Hepes pH 7.4, 500 mM NaCl and 1 mM TCEP. Both ion exchange and gel permeation purifications were performed at room temperature. Fractions were analysed for protein levels (comparing to standard concentrations of BSA) and purity by SDS-PAGE, using Sypro Ruby staining and fluorescence imaging as above. Remaining solution from each fraction was flash-frozen in liquid nitrogen after addition of 20% glycerol and stored at -80°C until further use.

Protein purity was calculated from SDS-PAGE scans using ImageJ (Gel Analysis Tool).

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